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<p>(21) International Application Number: PCT/GB92/00561 (22) International Filing Date: 27 March 1992 (27.03.92) (30) Priority data: 9106568.0 27 March 1991 (27.03.91) GB (71) Applicant (<i>for all designated States except US</i>): THE WELL- COME FOUNDATION LIMITED [GB/GB]; Unicorn House, 160 Euston Road, London NW1 2BP (GB). (72) Inventor; and (75) Inventor/Applicant (<i>for US only</i>): CHARLES, Ian, George [GB/GB]; The Wellcome Foundation Limited, Langley Court, Deckenham, Kent BR3 3BS (GB). (74) Agents: WOODS, Geoffrey, Corlett et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).</p>		<p>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.</p> <p>Published <i>With international search report.</i></p>
<p>(54) Title: BORDETTELLA BRONCHISEPTICA OUTER MEMBRANE ANTIGEN</p> <div style="text-align: right;">(I)</div> <p>DWNNQSI IKAGERQHGIHIKQSDGAGVRTATGTTIKVSGRQAQGVLLENPAEELRFQNG SVTSSGQLFDEGVRRFLGTVTVKAGKLVDHATLANVSDTRDDGIALYVAGEQAQASI ADSTLQGAGGVVRVERGANVTVQRSTIVDGGHLIGTLQPLQPEDLPSPRVVLGDTSVTAV PASGAPAAVS VF GANELTVDGGHITGGRAAGVAAMDGAIVHLQRATIRRGDAPAGGAVP GGA VP GGFG PLLDGWYGVDVSDSTVDLAQSIVEAPQLGAAIRAGR GAR V TVSGGSLSAP HG NV IETGGGAR RFP PPASPLSITLQAGARA QGRALLYRVLP EPVKLTLAGGAQQGDI VATELPPIPGASSGPLDVALASQARWTGATRAVDSL SIDNATWVM TDNSNV GALRLASD GSVDFQQPAEAGRFKCLMVD TLAGSGLFR MNVFADLG LSKLVVMRDASGQH RL LV RNS GSE PASGN TM LLVQT PRGSAATFTLANKDGKVDIGTYRYRLAANGNQWSLVGAKAPPA PKPAPQPGPQPPQPPQPPQPPQPPQRPPEAPAPQPPAGRELSAAAANA AVNTGGVGLAS TLWYAESN</p> <p>(57) Abstract</p> <p>A protein which is uncontaminated by components from <i>B. bronchiseptica</i>, which is capable of binding to antibody which also binds the native P.68 antigen of <i>B. bronchiseptica</i> and which has (a) the amino acid sequence (I) or (b) an amino acid sequence which has a homology of more than 98 % with the said amino acid sequence (a).</p>		

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BORDETELLA BRONCHISEPTICA OUTER MEMBRANE ANTIGEN

This invention relates to proteins suitable for use in vaccination against Bordetella bronchiseptica.

Bordetella bronchiseptica is a bacterial pathogen associated with respiratory diseases in animals, particularly atrophic rhinitis in pigs. The disease state is recognised by severe changes in the nasal architecture of developing piglets, pneumonia and growth retardation. We have demonstrated that protection against B.bronchiseptica mediated atrophic rhinitis correlates with the presence of an outer membrane protein with a molecular weight of 68kDa (P.68) as determined by SDS polyacrylamide gel electrophoresis (Novotny et al., 1985b). Antibodies to this 68kDa protein can be detected in high titre in protected piglets, while the titre is low or absent from non-protected animals (Novotny et al., 1985b). Furthermore, a naturally occurring mutant B.bronchiseptica lacking the 68kDa protein is unable to protect piglets when used as a vaccine and is unable to induce pathological changes in infected piglets (Novotny et al., 1985b).

Administration of a vaccine consisting of a purified preparation of the 68kDa protein to pregnant sows results in significant protection of the resulting piglets when challenged with virulent B.bronchiseptica (Kobisch & Novotny, 1990). In addition it has been demonstrated that passively administered monoclonal antibody BB05 (Novotny et al., 1985a) specific for the 68kDa protein is able to prevent death from pneumonia and development of atrophic rhinitis in mice that had been aerosol infected with a virulent strain of B.bronchiseptica.

Native P.68 antigen can only be purified at low levels from cultures of B.bronchiseptica. We have now found that the gene from which P.68 is expressed in fact encodes a protein with a Mr of 93996 (P.94). It may be surmised that P.94 is processed to form P.68 on the surface of B.bronchiseptica. We have also found that heterologous

expression of the full-length gene encoding P.94 in E.coli results in similar processing, with the P.68 antigen targeted to the bacterial outer membrane. This allows P.68 to be produced in large amounts uncontaminated by other B.bronchiseptica components.

Accordingly, the present invention provides a protein which is uncontaminated by components from B. bronchiseptica, which is capable of binding to antibody which also binds the native P.68 antigen of B.

10 bronchiseptica and which has (a) the amino acid sequence:
DWNQSQSIKAGERQHGIHIKQSDGAGVRTATGTTIKVSGRQAQGVLLENPAEELRFQNG
SVTSSGQLFDEGVRRFLGTVTVKAGKLVDHATLANVSDTRDDDGIALYVAGEQAQASI
ADSTLQAGGVRVERGANVTVQRSTIVDGGHLIGTLQPLQPEDLPSPRVVLGDTSVTAV
PASGAPAAVSVFGANELTVDGGHITGGRAAGVAAMDGAIVHLQRATIRRGDAPAGGAVP
15 GGAVPPGGFGPLLGDWYGVDVSDSTVDLAQSIVEAPQLGAAIRAGRGARVTVSGGSLSAP
HG NVIETGGGARFPFPASPLSITLQAGARAQGRALLYRVLPFVKLTLAGGAQQGQDI
VATELPPPIPGASSGPLDVALASQARWTGATRAVDLSIDNATVWMTDNNSNVGALRLASD
GSVD FQQPAEAGRFKCLMVD TLAGSGLFRMN VFADLG LSDKLVVMRDASGQHRLLRNS
GSE PASGN TM LLVQT PRGSAATFT LANKD GKVD IGTYRYRLAANGNQWSLVGAKAPPA
20 PKPAPQPGPQGPQPPQPPQPPQPPQPPQPEAPAPQPPAGRELSAAANA AVNTGGVG LAS
TLWYAESN

or (b) an amino acid sequence which has a homology of more than 98% with the said amino acid sequence (a).

The protein may be isolated and purified. It may therefore be provided in pure form. Purity may be 90% or more, or 95% or more. Amino acid sequence (a) is the amino acid sequence P.68. Amino acid sequence (b) may contain one or more amino acid insertions, deletions or substitutions. Amino acid sequences (a) and (b) may therefore differ from each other at no more than twelve positions, for example at no more than eight positions or at no more than four positions. The homology between the sequences may therefore be 98% or more, for example 99% or more.

35 One or more of the amino acid residues of amino acid sequence (a) may therefore be deleted or substituted or one

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or more additional amino acid residues may be inserted, provided the physicochemical character of the original sequence is retained for example in terms of charge density, hydrophobicity/hydrophilicity, size and configuration. A protein composed of the modified amino acid sequence must also be capable of binding to antibody which is capable of binding the native P.68 antigen.

Candidate substitutions are:

A for G and vice versa;

10 V by A, L or G;

K by R;

S by T and vice versa;

E by D and vice versa and

Q by N and vice versa.

15 A protein of the invention is obtained by recombinant DNA technology. The preparation of the protein therefore depends upon the provision of a DNA sequence encoding protein. However, we have found that the protein of the invention is a processed form of a larger precursor protein
20 designated P.94. A DNA sequence encoding P.94 may also be used to express a protein of the invention. In further aspects, therefore, the invention provides:

- a DNA sequence which encodes a protein of the invention composed of amino acid sequence (a) or (b);
- 25 - a DNA sequence which consists essentially of the nucleotide sequence shown in Figure 1 from nucleotide 247 to nucleotide 2040;
- a DNA sequence which differs from the nucleotide sequence shown in Figure 1 from nucleotide 247 to
30 nucleotide 2040 at no more than twelve positions;
- a protein which as the amino acid sequence shown in Figure 1 or an amino acid sequence which has a homology of more than 98% with the amino acid sequence shown in Figure 1;
- 35 - a DNA sequence which encodes a protein as defined immediately above;

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- a DNA sequence which consists essentially of the nucleotide sequence shown in Figure 1 from nucleotide 145 to nucleotide 2877;
- a DNA sequence which differs from the nucleotide sequence shown in Figure 1 from nucleotide 145 to 2877 at no more than twelve positions;
- a DNA sequence which consists essentially of the sequence shown in Figure 1 or a sequence which has a homology of more than 98% with the sequence shown in Figure 1.

The nucleotide sequences shown in Figure 1 from nucleotide 247 to 2040 and from nucleotide 145 to 2877 encode P.68 and P.94 respectively. DNA sequences which contain differences from these two sequences may have differences at up to twelve nucleotide positions, for example at no more than eight positions or at no more than four positions. These differences may or may not result in coding changes. Codons may be inserted or omitted, and nucleotides may be substituted. There may be a degree of homology of 98% or more, for example 99% or more, between the sequences shown in Figure 1 which encode P.68 and P.94 and modified versions of these DNA sequences.

A DNA sequence may be purified and isolated. A DNA sequence may be synthesised de novo, for example by synthesising and annealing oligonucleotides of appropriate sequences. A DNA sequence may be a cloned sequence.

The cloning of the DNA sequence may be carried out using standard procedures known in the art. However, it is particularly advantageous in such procedures to employ the sequence data disclosed herein so as to facilitate the identification and isolation of the desired cloned DNA sequences. Preferably, the DNA is isolated by the method described in Hull et al. (1981) as modified by Maskell et al., J. Bacteriol. 170: 2467-2471 (1988). The DNA is then digested with a restriction enzyme to generate short fragments which are then inserted into a cloning vector,

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such as the cosmid pH79 or a derivative thereof and the resulting recombinant DNA molecules used to transform E.coli and thus generate the desired library.

The library may be screened using a standard
5 screening strategy. For example one may employ as hybridisation probes one or more labelled oligonucleotides synthesised using the DNA sequence information disclosed herein. One or more additional rounds of screening of one kind or another may be carried out to characterise and
10 identify positive clones.

Having identified a first positive clone, the library may be rescreened for additional positive clones using the first clone as a hybridisation probe. Alternatively or additionally, further libraries may be prepared and these
15 may be screened using hybridisation probes. In this way, further DNA sequences may be obtained.

Thus cloned or synthesised, the desired DNA sequences may be inserted into an expression vector using known and standard techniques. The expression vector is normally cut
20 using restriction enzymes and the DNA sequence inserted using blunt-end or staggered-end ligation. The cut is usually made at a restriction site in a convenient position in the expression vector such that, once inserted, the DNA sequence is under the control of the elements of DNA that
25 effects its expression.

These elements may vary according to the host but usually include a promoter, ribosome binding site, translation start and stop sites, and a transcriptional termination site. Examples of such vectors include
30 plasmids and cosmids. Expression vectors of the present invention encompass both extrachromosomal vectors and vectors that are integrated into the host cell's chromosome.

The invention therefore also provides
35 - an expression vector which contains a DNA sequence as herein defined, and which, when provided in a

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suitable host, is capable of expressing encoded protein of the invention; and

- a host transformed with such an expression vector.

Examples of host cells of use with the invention
5 include prokaryotic and eukaryotic cells, such as bacterial, yeast, mammalian and insect cells. Particular examples of such cells are E.coli, S.cerevisiae, P.pastoris, Chinese hamster ovary and mouse cells, and Spodoptera frugiperda and Tricoplusia ni. The choice of
10 host cell may depend on a number of factors but, is post-translational modification of the antigen is important, then a prokaryotic host would be preferred.

Transformation of a host cell may be carried out using standard techniques. A phenotypic marker is usually
15 employed to distinguish between the transformants that have successfully taken up the expression vector and those that have not. Culturing of the transformed host cell and isolation of the antigen may also be carried out using standard techniques.

20 The invention therefore provides a process for the preparation of a protein of the invention, which process comprises maintaining a host transformed with an expression vector according to the invention under such conditions that the said protein is expressed. In more detail, the
25 process may comprise:

- cloning or synthesising a DNA sequence encoding the protein as herein defined;
- inserting the DNA sequence into an expression vector such that it is capable in an appropriate host of
30 being expressed;
- transforming a host cell with the expression vector;
- culturing the transformed host cell; and
- isolating the protein.

In this way heterologous expression of P.68 or a
35 modified version thereof according to the invention, or of P.94 or a modified version thereof according to the

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invention, may be achieved. These proteins may be expressed therefore in a host which is not B.bronchiseptica. The proteins can thus be obtained free of any other components of B.bronchiseptica. The P.94 or
5 modified version thereof may be processed to P.68 or a modified version thereof. P.68 or a modified version thereof can therefore be obtained from a host cell transformed with an expression vector containing a DNA sequence encoding P.68 or a modified version thereof or a
10 DNA sequence encoding the precursor molecule P.94 or a modified version thereof.

The protein obtained in this way may be insoluble and thus may need to be refolded following the use of guanidinium hydrochloride as denaturant in conventional
15 manner and in any event is preferably purified.

The invention additionally provides a veterinary composition comprising a protein of the invention and a veterinarily acceptable carrier or diluent. This composition may be used as a vaccine. The vaccine of the
20 invention may optionally contain additional antigens of B.bronchiseptica.

The vaccine of the invention is normally associated with a veterinarily acceptable vehicle which allows the protein to be administered to an animal. Administration is
25 usually carried out via the oral, intranasal, or preferably parenteral route. In the case of the parenteral route, the vehicle is generally liquid and the antigen is generally dissolved or suspended in it. An example of a liquid vehicle is physiological saline solution.

30 The vaccine may also contain an adjuvant for stimulating the immune response and thereby enhancing the potency of the antigen. Convenient adjuvants for use in the present invention include, for example aluminium hydroxide and aluminium phosphate.

35 Conveniently the vaccine contains a final concentration of protein in the range of from 0.01 to

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5mg/ml, preferably 0.03 to 2mg/ml, most preferably 0.3mg/ml. After formulation the vaccine may be incorporated into a sterile container which is then sealed and stored at a low temperature, for example 4°C, or is freeze dried.

The invention also provides a method for inducing immunity to B.bronchiseptica in animals, comprising the administration to an animal of an effective amount of a protein of the present invention. The antigen of the invention may therefore be used to induce immunity to B.bronchiseptica - induced atrophic rhinitis in pigs. In order to induce immunity one or more doses of the vaccine are normally administered. Each dose of the vaccine may be from 1ml to 5ml, preferably 2 to 4ml.

The following Examples illustrate the invention. In the accompanying Figures:

Figure 1 shows the DNA sequence of the prn gene encoding the P.68 pertactin from B.bronchiseptica. Downward arrows indicate the sites of two possible protein cleavage regions that result in the production of the mature polypeptide. Arrows over protein sequence denote two protein repeat motifs: (i) (GGXXP)₃ encoded by nucleotides 939-983 and (ii) (PQP)₇ encoded by nucleotides 1852 and 1947. Two RGD tripeptide sequences encoded by nucleotides 922-930 and 2245-2253 are shown overlined although only the first sequence occurs in the mature protein. An inverted repeat that is followed by a run of T-residues is shown between residues 2898-2909 and resembles a rho-independent transcription terminator.

Figure 2 is a line drawing representing the strategy used to generate the P.68 expression plasmid pBD881. Plasmids pBD875 and pBD856 contain the 5' and 3' sections of the gene encoding B.bronchiseptica prn respectively and were used to generate the full-length gene encoding P.94. This full-length (non-expressing) gene harboured by pBD876 was excised by digestion with AflIII and HindIII and ligated

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with NcoI-HindIII digested pKK233-2 so as to generate the P.68 expressing plasmid pBD881.

EXAMPLE

5 1. Methods

Bacterial strains, plasmids and phage. B.

B. bronchiseptica strain CN7531, was from the Wellcome culture collection (Wellcome Biotech, UK). E. coli K12 strains TG1 and HB101 were as previously described (Carter et al., 1985; Boyer & Roulland Dussoix, 1969). Cosmid pHC79 (Hohn & Collins, 1980) was from Amersham International (Little Chalfont, Buckinghamshire, UK). Plasmid pKK233-2 (Amann & Brosius, 1985) and M13 mp18 and M13 mp19 (Yanisch-Perron et al., 1985) were supplied by Pharmacia (Milton Keynes, Buckinghamshire, UK). E. coli strains were grown in Luria broth (LB) or LB solidified with 1-6% (w/v) agar (Millar, 1972). B. bronchiseptica was grown in Stainer-Scholte (Stainer & Scholte, 1962) broth or medium as previously described (Novotny et al., 1985a).

20 DNA isolation and manipulations. B. bronchiseptica CN7531 chromosomal DNA was prepared as described previously (Hull et al., 1981). Plasmid and bacteriophage DNA were isolated and purified by standard methods (Maniatis et al., 1982). All DNA-modifying enzymes were from Gibco BRL (Paisley, Scotland).

Construction of a B. bronchiseptica genomic library.

Cosmid pHC79 DNA was digested with BamHI and ligated with Sau3A digested B. bronchiseptica DNA in the 40-50kb size range as described previously (Charles et al., 1990). The gene bank was plated out and 480 randomly selected colonies transferred to microtitre plates as described previously (Charles et al., 1990). Colonies were transferred to Gene Screen plus hybridization membranes (Du Pont, Stevenage, Hertford, UK), and hybridized with a ³²P-labelled ClaI fragment of the prn gene encoding the P.70 protein from B. parapertussis (International Application No.

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PCT/GB91/02302) as described previously.

Subcloning and DNA sequencing. Recombinant phage DNA was sequenced after ligation of specific restriction endonuclease fragments of cosmid into the vector M13 mp18 and M13 mp19. Sequencing was carried out using universal primer [³⁵S] dATP and both gradient and wedge gels (Biggin *et al.*, 1983; Sanger *et al.*, 1977). Some clones were sequenced with modified T7 DNA polymerase (Tabor & Richardson, 1987) and 7-deaza-2-dGTP (Mizusawa *et al.*, 1986) using a kit supplied by Pharmacia. Gaps in the sequence were filled in using synthetic oligonucleotides made on a MilliGen 7500 DNA synthesizer (Millipore, UK) as specific primers (Charles *et al.*, 1986).

Immunological characterization of recombinant P.68.
15 E. coli lysates harbouring recombinant P.68 protein were subjected to SDS-PAGE electrophoresis (Maniatis *et al.*, 1982) and were transferred to nitrocellulose (Towbin *et al.*, 1979). The monoclonal antibody BB05 (Montaraz *et al.*, 1985; Novotny *et al.*, 1985a) was used to detect P.68
20 protein using horse-radish peroxidase conjugated goat anti-mouse second antibody and 4-chloro-1-naphthol as substrate (Fairweather *et al.*, 1986). To test for surface expression of recombinant P.68, slide agglutination assays were carried out as described in International Application No.
25 PCT/GB91/02302. More specifically, samples of E. coli strains to be tested (10^6 - 10^7) were mixed with 30 μ l of IgG purified polyclonal rabbit anti-P.69 antibody on a glass microscope slide and visually scored for clumping after 2 mins as compared with a negative control of either vir B. parapertussis or E. coli TG1.
30

2. Results

Isolation of the gene encoding the P.68 antigen. The B. bronchiseptica genomic library was screened by
35 hybridisation with a radioactively labelled 1.8kb ClaI fragment of the prn gene encoding the P.70 antigen from

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B.parapertussis. Of the two cosmids returning a positive signal one, designated pBD844, was selected for further analysis. Restriction mapping and Southern blotting experiments carried out on pBD844 demonstrated that the

5 ClaI and SalI hybridization pattern of the prn gene from B.bronchiseptica was identical to that described for both B.pertussis (Charles et al., 1989) and B.parapertussis (International Application No. PCT/GB91/02302). These ClaI and SalI fragments were therefore assumed to contain the

10 entire prn gene encoding the P.68 protein and were cloned into Bluescript pBSSKII⁺ to generate pBD875 (ClaI) and pBD856 (SalI); they were also cloned into M13 mp18 and M13 mp19 for DNA sequencing.

Nucleotide sequence of the prn gene encoding P.68 DNA

15 sequence was obtained initially from the ClaI and SalI fragments cloned into M13 and using universal primer; overlapping sequence was compared using a set of synthetic oligonucleotides as specific sequence primers. Computer analysis of the DNA sequence (Fig. 1) demonstrates that the

20 open reading frame for the prn gene of P.68 protein is capable of encoding a protein with a Mr of 93,996 (P.94). The molecular weight of the mature protein found on the surface of B.bronchiseptica is however 68,000 as judged by SDS-PAGE, suggesting that this molecule is the processed

25 form of the larger precursor. This processing is likely to involve the removal of both N-terminal and C-terminal sequences. N-terminal protein sequencing of purified preparations of P.68 from B.bronchiseptica gave the

30 sequence Asp-Trp/Gln-Asn-Asn-Gln-Gln/Ser-Ile-Xaa-Lys-Ala confirming that a signal peptide is cleaved. Cleavage of this signal peptide, of between 32 or 34 amino acid residues depending on which ATG initiation codon is used, occurs after the sequence AYA which is in agreement with the Ala-Xaa-Ala motif reported to be recognized by E. coli

35 signal peptidases (Perlman & Halvorson, 1983). Towards the C-terminus of the P.68 protein at residues 600-602 is a

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dibasic pair of amino acids, Lys-Arg, that occur in the same position, and are flanked by identical residues.

Examination of the first 140bp of the DNA sequence in Fig. 1 shows that there are no good fits with either the
5 consensus ribosome binding site (Shine & Dalgarno, 1975) or consensus promoter elements identified for *E. coli* genes (Rosenberg & Court, 1979). There is an inverted repeat after the stop codon, that is followed by a run of T-residues.

10 Heterologous expression of P.68 in *E. coli*. Cosmid pBD844 harbouring the *B. bronchiseptica* prn gene was unable to direct the expression of P.68 in *E. coli*. In order to express the gene in *E. coli* the entire structural gene was cloned into the expression plasmid pKK233-2. Fig. 2
15 outlines the expression strategy; an EcoRI-EcoRV fragment comprising the 5' end of P.68 prn from plasmid pBD875 was ligated into EcoRV-EcoRI digested pBD856 (containing the 3' end of P.68 prn) so as to generate pBD876 containing the entire structural gene. Plasmid pBD876 was digested with
20 AflIII-HindIII and ligated with NcoI-HindIII digested pKK233-2. This ligation mixture was used to transform *E. coli* TG1 and transformants expressing P.68 were identified by their ability to cross-react with the mAb BB05 in protein dot blots.

25 Colonies returning a positive signal were isolated and plasmid minipreps carried out to verify that a full-length insert has been cloned. One such plasmid, pBD881, was selected for further study. A Western blot of an SDS-PAGE gel of *E. coli* TG1 harbouring pBD881 surprisingly did
30 not produce a detectable 94kDa higher molecular weight band, but instead produces a pair of stronger bands at around 69kDa and 68kDa.

To test whether or not the P.68 protein was expressed on the surface of *E. coli*, slide agglutination assays were
35 carried out using both monoclonal antibody BB05 and polyclonal anti-P.69 antibody. Agglutination occurred just

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as rapidly with E. coli harbouring pDB881 as with vir⁺ B. bronchiseptica, suggesting that the heterologous protein is surface located.

5 REFERENCE EXAMPLE

Cloning of B. parapertussis chromosomal DNA into cosmid pHC79 and identification of the prn gene encoding P.70

B. parapertussis chromosomal DNA (prepared by the method of Hull et al, 1981 as modified by Maskell et al, 1988) was partially digested with Sau3A, and fragments in the 40-50kb size range were ligated into the BamHI site (Maniatis et al, 1982) of cosmid pHC79 (Hohn & Collins, 1980). Recombinant cosmids in E. coli HB101 were plated out and transferred to microtiter plates following the method described by Charles et al, 1990 and transferred to Gene Screen Plus hybridized membranes (Du Pont, Stevenage, Hertfordshire). The cosmids were then screened for the presence of the prn gene encoding P.70 by DNA : DNA hybridization using a radioactively labelled 1.8kb Clal restriction fragment isolated from the related prn gene from B. pertussis. The Clal fragment was gel purified (Tautz & Renz, 1983) following digestion of cosmid pI69 (Charles et al, 1989). The fragment was nick translated with a kit supplied by BCL, Mannheim and [$\alpha^{32}\text{P}$]-ATP (Amersham), and hybridized with the B. parapertussis gene bank filters as previously described (Charles et al, (1990)). Three positive colonies were identified and one, harbouring cosmid pBD811 was selected for further analysis.

30 Subcloning and DNA sequencing

Restriction fragments of cosmid pBD811 were cloned in M13mp18 and M13mp19 (Yanisch-Perron et al, 1985) and sequenced using universal primer, [$\alpha^{35}\text{S}$] dATP (deoxyadenosine 5'-($\alpha^{35}\text{S}$) thiotriphosphate) dideoxynucleotide triphosphates, and both gradient and wedge gels (Biggin et al, 1983; Sanger et al, 1977). To

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resolve compression artifacts some clones were sequenced with modified T7 DNA polymerase (Tabor & Richardson, 1987) and 7-deaza-2'-dGTP (Mizusawa et al, 1986) using a kit supplied by Pharmacia. Gaps in the sequence were filled in
5 using synthetic oligonucleotides as specific primers (Charles et al, 1986).

Oligonucleotides for use as specific sequencing primers were made on a SAM1 oligonucleotide synthesizer (Biolabs).

10 Computer analysis of the DNA sequence revealed an open reading frame capable of encoding a protein of 922 amino acids with a calculated molecular weight of 95,177.

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5 bronchiseptica and which has (a) the amino acid sequence:

or (b) an amino acid sequence which has a homology of more than 98% with the said amino acid sequence (a).

3. A DNA sequence according to claim 2, which consists essentially of the nucleotide sequence shown in Figure 1 from nucleotide 247 to nucleotide 2040.

5. A protein which as the amino acid sequence shown in Figure 1 or an amino acid sequence which has a homology of more than 98% with the amino acid sequence shown in Figure 1.

7. A DNA sequence according to claim 6, which
35 consists essentially of the nucleotide sequence shown in
Figure 1 from nucleotide 145 to nucleotide 2877.

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8. A DNA sequence according to claim 6, which differs from the nucleotide sequence shown in Figure 1 from nucleotide 145 to 2877 at no more than twelve positions.

5 9. A DNA sequence which consists essentially of the sequence shown in Figure 1 or a sequence which has a homology of more than 98% with the sequence shown in Figure 1.

10 10. An expression vector which contains a DNA sequence as defined in any one of claims 2 to 4 and 6 to 9, and which, when provided in a suitable host, is capable of expressing a protein as defined in claim 1 or 5.

11. A vector according to claim 10, which is a plasmid.

15 12. A host transformed with an expression vector as defined in claim 10 or 11.

13. A host according to claim 12, which is a strain of E. coli.

20 14. A process for the preparation of a protein as defined in claim 1, which process comprises maintaining a host as claimed in claim 12 or 13 under such conditions that said protein is expressed.

25 15. A process for the preparation of a protein as defined in claim 5, which process comprises maintaining a host as claimed in claim 12 or 13, which has been transformed by an expression vector as claimed in claim 10 which contains a DNA sequence as defined in any one of claims 6 to 9, under such conditions that the said protein is expressed.

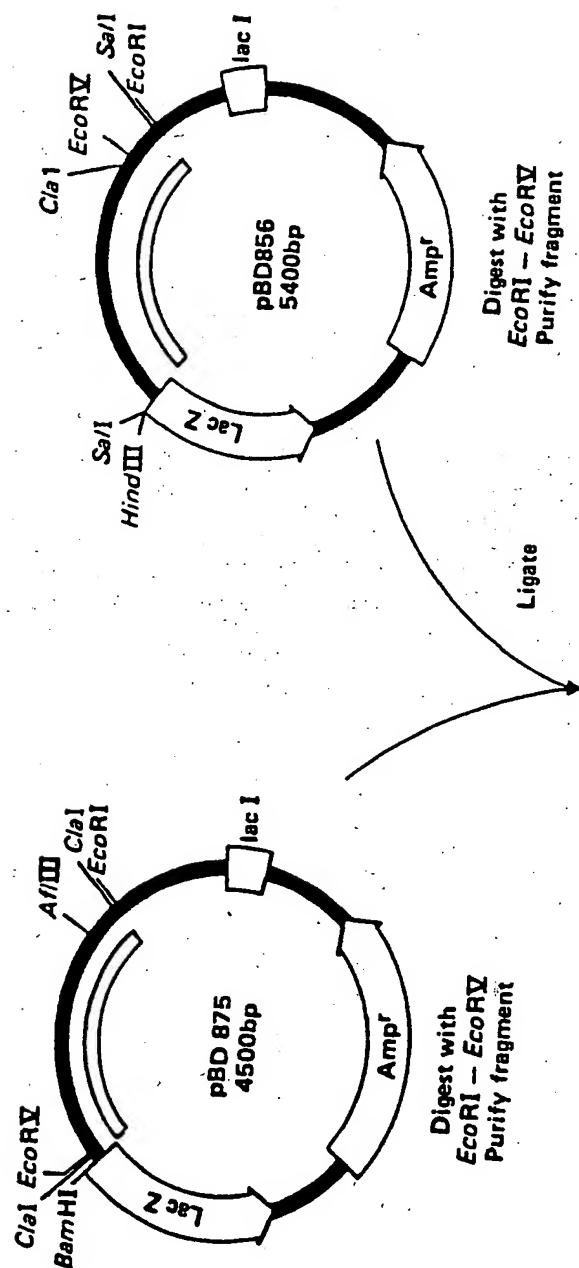
30 16. A veterinary composition comprising a veterinarily acceptable carrier or diluent and, as active ingredient, a protein as defined in claim 1 or 5.

Fig.1.(3/3)

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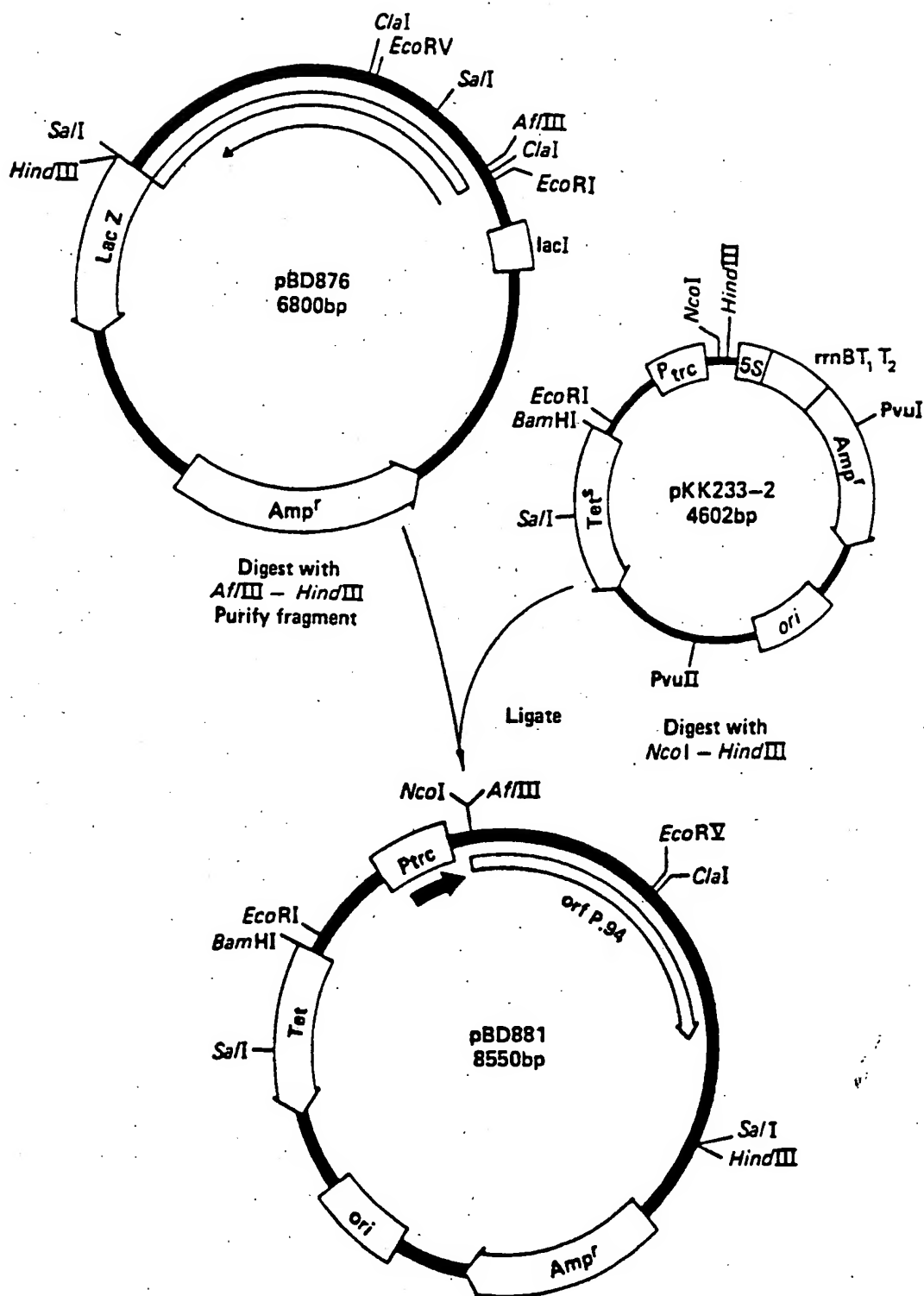
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Fig.2.



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Fig. 2. cont.



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 92/00561

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/31;	C07K13/00;	C12N1/21; A61K39/10
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	INFECTION AND IMMUNITY. vol. 47, no. 3, March 1985, WASHINGTON US pages 744 - 751; J. MONTAREZ ET AL.: 'Identification of a 68-kilodalton protective protein antigen from Bordetella bronchiseptica' see the whole document ---	1-16
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, 1989, WASHINGTON US pages 3554 - 3558; I. CHARLES ET AL.: 'Molecular cloning and characterization of protective outer membrane protein P.69 from Bordetella pertussis' see the whole document --- --/	1-16
<p>¹⁰ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
10 JUNE 1992	17 JUN 1992	
International Searching Authority	Signature of Authorizing Officer	
EUROPEAN PATENT OFFICE	VAN DER SCHAAL C.A.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	MOLECULAR MICROBIOLOGY vol. 5, no. 2, 22 February 1991, pages 409 - 418; L. LI ET AL.: 'P.70 pertactin, an outer-membrane protein from Bordetella parapertussis: Cloning, nucleotide sequence and surface expression in E. coli' see the whole document ---	1-16
P,X	WO,A,9 115 571 (THE WELLCOME FOUNDATION LTD) 17 October 1991 see the whole document ---	1-16

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 9200561
SA 57816**

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9115571	17-10-91	None	

10 FORM F077

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82